Bayesian estimation of the diagnostic accuracy of a multiplex real-time PCR assay and bacteriological culture for 4 common bovine intramammary pathogens

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ABSTRACT

Bacteriological culture (BC) is the traditional method for intramammary infection diagnosis but lacks sensitivity and is time consuming. Multiplex real-time PCR (mr-PCR) enables testing the presence of several bacteria and reduces diagnosis time. Our objective was to estimate bacterial species-specific sensitivity (Se) and specificity of both BC and mr-PCR tests for detecting bacteria in milk samples from clinical mastitis cases and from apparently normal quarters, using a Bayesian latent class model. Milk samples from 1,014 clinical mastitis cases and 1,495 samples from apparently normal quarters were analyzed by BC and mr-PCR. Two positive culture definitions were used: ≥ 1 cfu/0.01 mL and ≥ 10 cfu/0.01 mL of the specified bacteria. The mr-PCR was designed to simultaneously detect *Staphylococcus aureus*, *Streptococcus uberis*, *Escherichia coli*, and *Streptococcus agalactiae*. The priors used in our Bayesian model were weakly informative, with BC priors using the best available error data. Results were compared with those obtained using uniform priors for mr-PCR to test robustness. Weak and uniform priors gave about the same posterior distributions except for *Strep. uberis* from normal quarters and *Strep. agalactiae*. Multiplex real-time PCR Se on milk from clinical mastitis were lower than mr-PCR Se on milk from normal quarters. Multiplex real-time PCR Se was higher than BC on milk from normal quarters. Multiplex real-time PCR Se was generally lower than BC on milk from clinical mastitis and it varied by clinical severity.

The estimate specificities of detection for all pathogens were $\geq 99\%$, regardless of sample type. The effect of milk sample preservation before testing was evaluated and may have been a factor that affected our observed results. A significant association was observed between sample age and mr-PCR results leading to reduced detection of *E. coli* and *Strep. agalactiae* in nonclinical samples. Differences in sample age between conduct of BC and of mr-PCR did not concur with any apparent differences between Se estimates of the 2 tests. Further work should be done to extend these results to other PCR-based tests for detecting bacterial species in milk samples, for which presented results could be used as prior parameter distributions. Limits of sample handling and storage and the potential existence of substances in clinical case samples that may interfere with PCR reactions also are worth further investigation.

Key words: mastitis, bacterial culture, real-time polymerase chain reaction, latent class model

INTRODUCTION

Successful pathogen-specific mastitis research and implementation of effective farm-level mastitis control programs rely on accurate and timely diagnosis of clinical and nonclinical IMI. Bacteriological culture is a method in widespread use, but this testing method could lack sensitivity (Se), as a large proportion of milk samples taken from clinical or subclinical mastitis cases show no growth in conventional culture (Makovec and Ruegg, 2003; Olde Riekerink et al., 2008; Taponen et al., 2009). For example, a negative culture may result from low numbers of bacteria in the sample (Sears et al. 1990). Bacteriological culture requires incubation time and distinguishing definitively between morphologically similar pathogens may require subculture and biochemical testing and, hence, additional materials, labor, and time (Hogan et al., 1999).

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The financial advantages of mastitis diagnosis tools that provide better diagnostic properties for the cost are obvious. Therefore, interest is increasing in the use of PCR technology to diagnose IMI in dairy cows (Viguier et al., 2009). Of special interest are multiplex real-time PCR (**mr-PCR**) assays, which enable simultaneous testing for the presence of several different bacterial species in milk samples. The technology works by detecting the unique DNA composition of specific mastitis-causing bacteria in milk samples. Several mr-PCR assays are commercially available and several more are likely to be offered in the coming years. However, despite the potential attractiveness of possibly greater diagnostic Se and specificity (**Sp**), as well as potential reduced time to diagnosis, routinely using this technology is not yet advised. Prior to use in the field, the assay's false negative ($1 - \text{Se}$) and false positive ($1 - \text{Sp}$) rates need to be well estimated to avoid a substantial risk of erroneous farm-level decision making and subsequent failure to optimize udder health on the farm.

Direct calculation of the Se and Sp values presumes perfect knowledge of the true infection state of all tested samples. The limitations of bacteriological culture of milk samples render it imperfect as a true gold standard, despite its practical recognition as the reference method of choice (National Mastitis Council, 2004). To circumvent the problem of an imperfect gold standard, Bayesian latent class modeling can be used (Dohoo et al., 2009). Such a model uses the results of multiple tests applied to all samples to estimate the unknown (or latent) true infection state of each sample and, hence, the error rates for all tests simultaneously, without assuming any to be a perfect reference standard. Bayesian methods work through Bayes Theorem, which states that posterior distributions summarizing final conclusions are the product of prior information (summarizing predata knowledge) and the likelihood function, containing the information in the current data set. Through the use of prior information, Bayesian analysis can estimate more parameters than there are degrees of freedom in the data, which can be an advantage in diagnostic testing situations in which no perfect reference standards exist. This advantage comes at the cost of having to specify prior distributions, upon which the final conclusions may depend.

The National Cohort of Dairy Farms (**NCDF**) assembled by the Canadian Bovine Mastitis Research Network (**CBMRN**; Saint-Hyacinthe, QC, Canada) provides a well-defined Canadian dairy population (Reyher et al., 2011) in which to estimate the diagnostic accuracy of a recently developed mr-PCR assay that targets some of the common pathogens implicated in

IMI: *Staphylococcus aureus*, *Streptococcus uberis*, *Escherichia coli*, and *Streptococcus agalactiae*. Although *Strep. agalactiae* has become very uncommon, it is still quite important to recognize this pathogen when it is present to arrest its transmission as rapidly as possible (Keefe, 1997).

The aim of this research was to estimate, through Bayesian modeling, the bacterial species-specific Se (proportion of real bacterial species-specific infected quarters that test positive) and Sp (proportion of real bacterial species-specific uninfected quarters that test negative) values of both traditional bacteriological culture and an mr-PCR test as applied to clinical mastitis milk samples and in large-scale IMI screening samples.

MATERIALS AND METHODS

Data Sources

Quarter milk samples from 1,701 clinical mastitis cases (clinical samples) were acquired from all farms in the CBMRN Core Research Platform's NCDF through the Mastitis Laboratory Network (**MLN**; Reyher et al., 2011). These samples represent a census of the clinical cases identified during the 2007 data collection year of the NCDF. Also, 4,000 randomly selected mammary quarter milk samples that originate from randomly selected lactating cows and cows before dry-off and after calving that were all apparently not experiencing clinical mastitis (nonclinical samples) were acquired in the NCDF 2008 IMI sampling series.

The NCDF comprises 91 commercial dairy farms in 6 provinces sampled over a 2-yr period. Research centers in Alberta, Ontario, Quebec, and the Maritime provinces (Prince Edward Island, Nova Scotia, and New Brunswick) were established by regional directors of the CBMRN (Reyher et al., 2011). The NCDF farms were primarily Holstein-Friesian herds participating in DHI milk recording and were selected to achieve a uniform distribution among 3 strata of bulk tank SCC and to reflect their respective provinces' proportions of freestall housing systems.

Standardized protocols were implemented for milk samplings, sample handling, and data collection (protocols available upon request), and training of producers to follow protocols. For clinical cases of mastitis, farmers were asked to sample cows they identified as having abnormal milk or more severe clinical signs. Single samples of the affected quarter were taken on the day of diagnosis, and repeated at 2 to 3 and at 4 to 5 wk after diagnosis. Milk samples for detecting bacteria in the milk of apparently normal cows were obtained from the longitudinal incidence-density sampling series

described in Reyher et al. (2011). The sampling was directed at lactating cows with no abnormal milk at the beginning of the series. These samples were taken by trained research technicians. Fifteen cows were enrolled for 4 separate time periods throughout the 2 yr.

Milk samples taken by farm personnel were frozen and stored on the farm until monthly pickup by technicians. Samples taken by technicians were placed on ice and subsequently frozen for storage at -20°C . Samples were submitted to 1 of 4 milk bacteriology laboratories comprising the MLN. The MLN represents coordinated regional mastitis bacteriology laboratories using standardized procedures in which bacteriological culture and species identification was developed based on National Mastitis Council guidelines (Hogan et al., 1999). For details on milk sample processing, please see NCDF (2009). After bacterial identification, 2-mL aliquots of all clinical case samples were placed into 2 sterile 2.0-mL CryoVials (Nalgene no. 5012-0020; Nalgene, Rochester, NY), frozen, and shipped on ice by overnight courier to the CBMRN central laboratory and stored at -80°C for later use for mr-PCR testing. Samples from nonclinical mammary quarters were selected systematically as every eighth sample received by each laboratory and were handled in the same manner as clinical case samples. All bacteriological data were stored in a relational database (Access 2003; Microsoft Corp., Redmond, Washington). For more details, please refer to Reyher et al. (2011).

Each of the 1,701 clinical and 4,000 nonclinical samples previously stored in CryoVials were transferred to the collaborating laboratory of Steve Oliver at The University of Tennessee (Knoxville) for identification of bacteria by the mr-PCR assay developed in his laboratory. The assay was designed to simultaneously detect 4 mastitis pathogens in bovine milk: *Staph. aureus*, *Strep. uberis*, *E. coli*, and *Strep. agalactiae*. However, among the 4,000 nonclinical samples, only 1,500 were tested by mr-PCR, as the estimated prevalence of mr-PCR-positive samples was low and simulation of Bayesian estimations with greater sample sizes indicated that very little additional precision of estimates would be obtained by continuing to analyze nonclinical samples. Consequently, the tested nonclinical samples only originated from Alberta and Ontario regions because of systematic region testing order. The mr-PCR bacterial identification data were linked to the CBMRN NCDF bacterial culture data for Se and Sp estimation.

Finally, culture and mr-PCR results from clinical milk samples that were not harvested the day of diagnosis, but 2 to 5 wk after diagnosis, were excluded from this study ($n = 612$). Samples whose laboratory results sheets had codes indicating laboratory accidents, damaged samples, samples with a doubtful sample type,

and samples with doubtful identification numbers were also eliminated from analysis ($n = 84$).

DNA Isolation and mr-PCR

Milk samples were enriched with Trypticase soy broth (Becton Dickinson and Co., Franklin Lakes, NJ) and incubated for 16 to 18 h at 37°C . After overnight enrichment, bacterial DNA was isolated directly from milk following the method described by Gillespie and Oliver (2005). American Type Culture Collection (ATCC, Manassas, VA) reference strains used as positive controls for the mr-PCR assay included *Staph. aureus* (ATCC 10832), *Strep. uberis* (ATCC 27958), *E. coli* (ATCC 25922), and *Strep. agalactiae* (ATCC 27956). Controls for the real-time PCR assay were prepared by inoculating UHT milk with 1 of the 4 ATCC control strains and performing DNA isolation as described by Gillespie and Oliver (2005). The mr-PCR developed in the Oliver laboratory targets the following genes: *nuc* gene for *Staph. aureus*, plasminogen activator gene for *Strep. uberis*, *wec A* gene for *E. coli*, and *cfb* gene encoding the Christie-Atkins-Munch-Petersen (CAMP) factor for *Strep. agalactiae*. Primers and dual-labeled probes for detection of *Strep. uberis* and *Strep. agalactiae* were as described by Gillespie and Oliver (2005). Primers and dual-labeled probes for *Staph. aureus* targeted the *nuc* gene based on primers used by Brakstad et al. (1992), and for *E. coli* the *wec A* gene based on primers used by Bayardelle and Zafarullah (2002). Primers and dual-labeled probes were purchased from Integrated DNA Technologies Inc. (IDT, Coralville, IA). The real-time PCR was performed as described by Gillespie and Oliver (2005) with modifications. Each real-time PCR run was performed in the iQ5 real-time PCR instrument (Bio-Rad Laboratories Inc., Hercules, CA). Samples were run in a 96-well plate in triplicate. Each 96-well plate contained, in triplicate, a nontemplate control, *Staph. aureus*-, *Strep. uberis*-, *E. coli*-, and *Strep. agalactiae*-positive controls. A total of 27 milk samples were run in triplicate on each plate. Milk samples were considered positive or negative based on their threshold cycle (C_t) value. The C_t is defined as the cycle number at which the fluorescence passes the threshold baseline. The C_t value determines if a sample is negative or positive based on the values of the positive controls and the nontemplate control. The negative cut-off C_t value was based on the average of the negative control C_t values and the negative sample C_t values. The average C_t value of this minus 1 standard deviation was used to establish the negative and positive values. The negative threshold values for *Staph. aureus*, *Strep. uberis*, *E. coli*, and *Strep. agalactiae* were 28.61, 28.33, 29.55, and 21.52, respectively.

Bayesian Estimation of Se and Sp of Bacterial Culture and mr-PCR

To estimate the Se and the Sp of both assay methods for each of the 4 specific bacterial species, we used the Bayesian latent class model developed by Joseph et al. (1995) applied to conditionally dependent tests (Dendukuri and Joseph, 2001). The model was implemented in the JAGS 2.2.0 program (Plummer, 2003) called through R 2.13.0 software (R Development Core Team, 2009) using the rjags package. A separate model was fit for each of the 8 combinations of sample type (clinical quarter or nonclinical quarter) and 4 bacterial species. Additional models were fit to explore the effects of certain subgroupings on Se and Sp estimates. Aside from the data, which consist of the cross-classification of results from the 2 tests, the model requires prior inputs for the 5 unknown parameters, including the prevalence of the bacterial species under study and the Se and Sp of each test. The a priori information, referred to as priors, was entered as β density functions, as is standard for binomial parameters (Joseph et al., 1995).

To create prior distributions for the Se and Sp of bacteriological culture for *Staph. aureus*, *Strep. uberis* and *E. coli* Se and Sp, the recent study by Dohoo et al. (2011) was used. Dohoo et al. (2011) estimated both the Se and Sp of a variety of definitions of IMI-based results from a single milk sample by using pseudo gold standard consensus-based IMI definitions obtained from a set of triplicate samples that had been taken at 1-wk intervals. Prior information for *Strep. agalactiae* came from Dinsmore et al. (1991), which is the most recent published work on the accuracy of cultures of single samples for the diagnosis of *Strep. agalactiae* IMI. Please refer to Table 1 to see all prior information used.

Several publications have reported some species-specific Se and Sp mr-PCR assay estimates on milk samples harvested from the field (Phuektes et al., 2001; Gillespie and Oliver, 2005; Koskinen et al., 2010; Wellenberg et al., 2010). However, in all these publications, the bacteriological culture was used as the gold standard for the calculations of the Se and Sp estimates, making these estimates potentially biased by the imperfect accuracy of bacteriological culture. Weak prior information were used for mr-PCR Se, captured with a β (a , 1) prior distribution increasing monotonically to a finite maximum of 1. This distribution favors larger Se (i.e., vague prior judgment that the test is reasonably good, or a reluctant prior of seeing the test as perfect). From Gillespie and Oliver (2005) who used the same mr-PCR as in this study, we determined the β distributions with a median set at 92% for all species except for *E. coli*. For *E. coli*, having no reference, we based our β distribution on the bacterial culture and, using

Table 1. Prior information and related β distribution parameters (a , b) used for Bayesian estimation of bacterial species-specific and culture definition-specific sensitivities (Se) and specificities (Sp) of bacteriological culture and a multiplex real-time PCR (mr-PCR) assay applied simultaneously to the same milk samples

Bacterial species	Culture-positive definition	Test	Se			β parameter			Sp			β parameter		
			Median Se	95% CI		a	b		Median Sp	95% CI		a	b	
<i>Staphylococcus aureus</i>	A ¹	Culture	0.90	(0.88, 0.93)		519.8	55.7		0.998	(0.997, 0.998)		11,462	24	
	C ²	Culture	0.72	(0.68, 0.76)		396.7	154.9		100	(0.9999, 1.0)	Same as culture	36,881	1	
<i>Streptococcus uberis</i>	A and C	mr-PCR	0.92	(0.65, 0.99)		8.4	1							
	A	Culture	0.87	(0.78, 0.92)		87.2	14.5		100	(0.999, 1.0)		3,687	1	
<i>Escherichia coli</i>	C	Culture	0.81	(0.72, 0.88)		80.6	19.9		100	(0.9998, 1.0)		18,445	1	
	A and C	mr-PCR	0.92	(0.65, 0.99)		8.4	1				Same as culture			
<i>Streptococcus agalactiae</i>	A	Culture	0.77	(0.50, 0.93)		11.9	4.4		0.999	(0.9985, 0.999)		22,980	24	
	C	Culture	0.47	(0.23, 0.72)		5.9	7.7		100	(0.9998, 1.0)		14,979	1	
<i>Streptococcus agalactiae</i>	A and C	mr-PCR	0.77	(0.24, 0.99)		2.6	1				Same as culture			
	A and C	Culture	0.98	(0.95, 1.0)		151.8	4.1		0.995	(0.97, 1.0)		138.7	1.7	
	A and C	mr-PCR	0.92	(0.65, 0.99)		8.4	1				Same as culture			

¹Presence of at least 1 cfu/0.01 mL of the bacteria in pure or mixed culture (Dohoo et al., 2011).

²Presence of at least 10 cfu/0.01 mL of the bacteria in pure or mixed culture (Dohoo et al., 2011).

the same approach, set the median at the same level of 77%. All prevalence priors had a flat β distribution with parameters (1, 1). Prior Se, Sp, and prevalence β distributions were estimated using BetaBuster (<http://www.epi.ucdavis.edu/diagnostictests/betabuster.html>).

To assess the effect of prior information on the posterior distributions, each model was estimated, but with uniform priors [β (1, 1)] for Se substituted for the above-described priors. The goal was to have posteriors that were not dependent on the exact functional form of the priors, the analysis being robust to the choice of priors (Albert, 2009). Also, the random number generator of Wichmann and Hill (1983) was specified as starting seed for each chain. To improve convergence and get more precise estimates of the posterior distribution, 2 Markov chain Monte Carlo chains were run with different starting values (Gelman and Rubin, 1992). For each chain, after a burn-in phase of 2,000 iterations, 50,000 iterations were monitored with a thinning of 1 in 10. If any convergence problems were identified with trace plot, autocorrelation plot, Gelman-Rubin test or Geweke's convergence diagnostic test, 50,000 additional iterations were monitored for each chain with a thinning of 1 in 25.

To see if the use of a different definition of positive diagnostic for culture has an effect on Se and Sp estimates, 2 definitions already considered in Dohoo et al. (2011) were evaluated: presence of at least 1 cfu/0.01 mL of the bacteria (culture definition A in Dohoo et al., 2011) or presence of at least 10 cfu/0.01 mL (definition C) in pure or mixed culture. Samples that had more than 2 bacterial species present according to the mr-PCR test or in culture were considered contaminated and excluded from analyses, except in the calculations for *Staph. aureus* and *Strep. agalactiae*, as in Reyher et al. (2011).

The quarters having clinical symptoms were given a severity score between 1 and 3 at the time of sampling by the person performing the sampling (Sears and McCarthy, 2003). Subgroups mild, moderate, and severe consisted of cows with abnormal milk only, cows with abnormal milk with swollen quarter, and cows with abnormal milk, swollen quarter, and systemic symptoms, respectively. Estimates of Se and Sp were made within each of these subgroups separately to explore potential associations of Se and Sp of bacterial culture and mr-PCR with mastitis severity.

Effect of Sample Age on mr-PCR Performance

The effect of the number of days from sample harvest to the performance of mr-PCR was evaluated by cross-tabulating mr-PCR pathogen-specific results (positive or negative) versus total time (d) between the date the

sample was harvested and the date the sample was tested by mr-PCR (clinical case samples: <450, 450–549, and 550 positive days; nonclinical samples: <250, 250–349, and 350 positive days) and a χ^2 (or Fisher exact) test was performed. The effect of sample age on the odds of a positive mr-PCR test of a pathogen was evaluated using generalized linear mixed models for a binomial family with a logit link in which sample age was put on a continuous scale, and region and season of sampling were also included as well as a random effect for herd. Parameter estimates were obtained by minimizing the Laplacian approximation to the deviance function with the lme4 package in R (Bates and al., 2011). Interaction terms between sample age and region were evaluated but no consistent regional patterns were observed. For practicality, therefore, only models without interactions are presented. The assumption of linearity of sample age effect was verified by dividing sample age into 4 categories that were roughly equal-sized and modeled in the form of indicator variables. The log odds of a positive mr-PCR test for each pathogen among the observations that fall in each of the 4 categories was computed and those values were plotted against the midpoints of the categories.

Effect of Intertest Time Interval

The time lapse between culture and mr-PCR tests was calculated by counting the number of days between the date the sample was cultured and the date the sample was analyzed by mr-PCR. To verify that the time difference was not associated with pathogen-specific discordant results between bacteriological culture and mr-PCR assay, for each type of sample and pathogen, the odds ratios of time difference on the risk of mr-PCR positive results among specific pathogen-negative cultures and specific types of samples were estimated using the same approach as above, with generalized linear mixed models in which time difference was put on a continuous scale, including region and season of sampling and random effects for herd.

RESULTS

Descriptive Data

In total, 1,013 clinical milks samples and 1,492 non-clinical samples were analyzed by both bacteriological culture and mr-PCR. The clinical samples were collected between January 31, 2007, and October 18, 2007. They were cultured between February 19, 2007, and November 6, 2007, and mr-PCR tested between January 17, 2008, and February 2, 2009. The nonclinical samples were collected between January 16, 2008, and

October 9, 2008. They were cultured between June 21, 2008, and December 2, 2008, and mr-PCR tested between February 12, 2009, and June 8, 2009.

The clinical milk samples came from 88 herds and the nonclinical milk samples came from 37 herds. Among the 88 herds from which clinical samples were collected, 16, 26, 28, and 18 herds were from Alberta, Ontario, Quebec, and Atlantic regions, respectively. Among the 37 herds from which nonclinical samples were taken, 14 and 23 herds were from Alberta and Ontario regions, respectively. Two hundred four, 343, 213, and 254 clinical samples collected from Alberta, Ontario, Quebec, and Atlantic regions, respectively. Five hundred fourteen and 981 nonclinical samples harvested from Alberta and Ontario regions, respectively. A median of 10 (SD = 8.9) clinical samples and 36 (SD = 15.7) nonclinical samples were taken per herd. Among the nonclinical samples, 385, 454, and 305 were the first, second, and third respective sequential intensive incidence-density samples from lactating cows, in either of 2 intensive sampling periods of 2008; 178 and 165 samples were collected between 4 and 2 wk before dry-off and between 2 wk and dry-off, respectively; 8 samples were taken within 2 wk after calving.

The bacterial culture and mr-PCR results for clinical and nonclinical samples are cross-classified in Table 2. Among clinical samples, the greater prevalence and bias corrected κ coefficient of agreement between culture and mr-PCR results was seen for *Strep. agalactiae* detection (>99%), followed by *Strep. uberis* (~94%)

and *Staph. aureus* (~91%). The least agreement was seen for *E. coli* detection (~85%). Among nonclinical samples, the agreement between culture and mr-PCR tests for *E. coli*, *Strep. uberis*, or *Strep. agalactiae* detection was quite similar (between 98 and 99%). The least agreement was seen for *Staph. aureus* detection (~95%). According to bacterial culture results, 191 subclinical samples showed greater than 2 species (contaminated samples). Among these 191 samples, 3 were positive for *E. coli* and one was positive for *Strep. uberis* at mr-PCR testing. According to mr-PCR results, only 1 subclinical sample showed greater than 2 species, and this sample showed 10 cfu/0.01mL of *Staph. aureus* at culture. Relating to clinical samples, 98 of these showed greater than 2 species at culture. Among these 98 samples, 12 were positive to *E. coli* and 11 were positive for *Strep. uberis* at mr-PCR testing. According to mr-PCR results, 3 clinical samples showed greater than 2 species. At culture, 2 of these 3 samples were negative and the other sample had *Staph. aureus* growing.

Se and Sp Estimates

Tables 3, 4, 5, and 6 present the posterior medians obtained with their 95% Bayesian credible intervals (BCI) for the estimated Se of mr-PCR and bacteriological culture using definitions A and C on clinical and nonclinical sample types to detect each of the 4 pathogens. For detecting *Staph. aureus* in milk samples from nonclinical quarters using culture-positive defini-

Table 2. Cross-classification of results of bacteriological culture and a multiplex real-time PCR (mr-PCR) for detecting bacteria in mammary quarter milk samples from farmer-identified clinical mastitis cases (clinical) and from randomly selected lactating cows that were apparently not experiencing clinical mastitis (nonclinical)

Bacterial species	Culture positive definition ¹ (cfu/0.01 mL)	Culture result	mr-PCR result			
			Clinical		Nonclinical	
			+	–	+	–
<i>Staphylococcus aureus</i>	1	+	95	41	36	13
		–	46	831	69	1,374
	10	+	84	37	31	6
		–	57	835	74	1,381
<i>Streptococcus uberis</i> ²	1	+	32	21	1	0
		–	34	825	11	1,288
	10	+	30	20	1	0
		–	36	826	11	1,288
<i>Escherichia coli</i> ²	1	+	64	84	0	4
		–	62	702	20	1,276
	10	+	50	49	0	0
		–	76	737	20	1,280
<i>Streptococcus agalactiae</i>	NA ³	+	0	0	0	0
		–	1	1,012	12	1,480

¹As in Dohoo et al. (2011).

²Calculations for *Strep. uberis* and *E. coli* excluded samples with greater than 2 species identified by culture or mr-PCR.

³No culture growth was observed for *Strep. agalactiae*.

tion A, the Se of mr-PCR was slightly higher than for culture, but with overlapping BCI (Table 3). The same was true when culture was interpreted by definition C, but the BCI did not overlap (Table 4). On clinical-case samples, bacterial culture interpreted with definition A had higher Se than mr-PCR (Table 3), whereas culture and mr-PCR Se were equivalent when culture was interpreted by definition C (Table 4). Within all 3 clinical mastitis severity levels, bacterial culture interpreted with definition A was mostly more sensitive than mr-PCR (Table 5), whereas when culture was interpreted with definition C, culture Se was lower than mr-PCR, but with overlapping credibility intervals in all clinical mastitis severity levels (Table 6).

For detecting *Strep. uberis* in milk samples, the estimated Se of bacterial culture was not different from the estimated Se of mr-PCR. This was true for samples from nonclinical or clinical mammary quarters, and for bacterial culture interpreted by definition A or definition C. As with *Staph. aureus* detection, the culture definition influenced the estimated Se of bacterial culture, as is expected, but had no apparent association with the estimated Se of mr-PCR.

With respect to detecting *E. coli* in nonclinical milk samples, the estimated Se of mr-PCR was substantially higher than that of bacterial culture, with no overlap of BCI (Table 3). For detecting *E. coli* in samples from clinical-case quarters, bacteriological culture-estimated Se was higher than mr-PCR-estimated Se, but the BCI were also overlapping (Table 3). For moderate and severe clinical-case samples, the estimated Se of bacterial culture was substantially higher than the estimated Se of mr-PCR (Table 5), but these differences disappeared when bacterial culture was interpreted by definition C (Table 6). As with *Staph. aureus* and *Strep. uberis* detection, the culture definition influenced the estimated Se of bacterial culture, as is expected, but had no apparent association with the estimated Se of mr-PCR.

With respect to *Strep. agalactiae*, differences in estimated Se relative to using mr-PCR or bacterial culture were not apparent. Estimates were essentially equivalent to prior values, which is a function of the small number of *Strep. agalactiae*-positive observations.

The Sp estimates, when using culture definition A or C, *Staph. aureus*, *Strep. uberis*, and *E. coli*, were all $\geq 99.7\%$ with a 95% BCI $< 0.3\%$, whatever the sample type. Because no culture growth occurred for *Strep. agalactiae*, the culture definitions were equivalent and Sp estimates were $\geq 99.1\%$ with a 95% BCI $< 1\%$.

Association of Sample Age with mr-PCR Se

No associations were observed between sample age and the frequency of positive mr-PCR results on clini-

Table 3. Posterior median and 95% Bayesian credible intervals (BCI) of bacterial species-specific and sample-type-specific sensitivity (Se) of bacterial culture and a multiplex real-time PCR (mr-PCR) assay using a 1 cfu/0.01 mL sample-positive definition (A in Dohoo et al., 2011)

Bacteria species	Sample type	Weak priors			Uniform priors		
		mr-PCR ¹		Culture	mr-PCR ¹		Culture
		Median Se	95% BCI	Median Se	95% BCI	Median Se	95% BCI
<i>Staphylococcus aureus</i>	Nonclinical ²	0.89	(0.81, 0.96)	0.83	(0.80, 0.86)	0.88	(0.79, 0.95)
	Clinical ³	0.75	(0.69, 0.82)	0.86	(0.83, 0.89)	0.74	(0.67, 0.81)
<i>Streptococcus uberis</i>	Nonclinical	0.95	(0.72, 0.99)	0.84	(0.75, 0.91)	0.79	(0.16, 0.99)
	Clinical	0.71	(0.61, 0.81)	0.71	(0.64, 0.78)	0.68	(0.57, 0.79)
<i>Escherichia coli</i>	Nonclinical	0.76	(0.45, 0.98)	0.33	(0.19, 0.50)	0.70	(0.38, 0.97)
	Clinical	0.42	(0.32, 0.51)	0.50	(0.38, 0.60)	0.41	(0.30, 0.50)
<i>Streptococcus agalactiae</i>	Nonclinical	0.92	(0.64, 0.99)	0.98	(0.94, 0.99)	0.51	(0.03, 0.98)
	Clinical	0.92	(0.63, 0.99)	0.98	(0.94, 0.99)	0.49	(0.02, 0.97)

¹Polymerase chain reaction threshold cycle (Ct) values for *Staph. aureus*, *Strep. uberis*, *E. coli*, and *Strep. agalactiae* were 28.61, 28.33, 29.55, and 21.52, respectively.

²Mammary quarter milk samples from randomly selected lactating cows that were apparently not experiencing clinical mastitis.

³Mammary quarter milk samples from farmer-identified clinical mastitis cases.

Table 4. Posterior median and 95% credible intervals (BCI) of bacterial species-specific and sample-type-specific sensitivity (Se) of bacterial culture and a multiplex real-time PCR (mr-PCR) assay using a 10 cfu/0.01 mL sample-positive definition (C in Dohoo et al., 2011)

Bacteria species	Sample type	Weak priors			Uniform priors		
		mr-PCR ¹		Culture	mr-PCR ¹		Culture
		Median Se	95% BCI		Median Se	95% BCI	
<i>Staphylococcus aureus</i>	Nonclinical ²	0.96	(0.88, 0.99)	0.66	0.95	(0.87, 0.99)	0.66
	Clinical ³	0.74	(0.66, 0.81)	0.69	0.72	(0.64, 0.79)	0.69
<i>Streptococcus uberis</i>	Nonclinical	0.96	(0.77, 0.99)	0.76	0.90	(0.23, 0.99)	0.77
	Clinical	0.71	(0.60, 0.81)	0.66	0.68	(0.56, 0.78)	0.66
<i>Escherichia coli</i>	Nonclinical	0.90	(0.58, 0.99)	0.18	0.87	(0.50, 0.99)	0.18
	Clinical	0.46	(0.28, 0.58)	0.36	0.44	(0.25, 0.57)	0.34
<i>Streptococcus agalactiae</i>	Nonclinical	0.92	(0.64, 0.99)	0.98	0.51	(0.03, 0.98)	0.98
	Clinical	0.92	(0.64, 0.99)	0.98	0.50	(0.03, 0.98)	0.98

¹Polymerase chain reaction threshold cycle (Ct) values for *Staph. aureus*, *Strep. uberis*, *E. coli*, and *Strep. agalactiae* were 28.61, 28.33, 29.55, and 21.52, respectively.

²Mammary quarter milk samples from randomly selected lactating cows that were apparently not experiencing clinical mastitis.

³Mammary quarter milk samples from farmer-identified clinical mastitis cases.

cal-case samples for *Staph. aureus*, *Strep. uberis*, or *E. coli* (too few observations for *Strep. agalactiae*). However, in samples from nonclinical quarters, an association existed between the number of samples with positive mr-PCR results for *E. coli* and *Strep. agalactiae* and sample age, but not for *Staph. aureus* and *Strep. uberis* (95% CI 0.02–0.47, 0.00–0.23, 0.47–1.51, and 0.16–4.3 respectively; Table 7). Herd-level variances averaged 0.58 (0.21–1.01).

Association of Intertest Time Interval with Culture and mr-PCR Results

The estimated odds ratios of a positive mr-PCR per day of intertest interval duration among culture negative samples are given in Table 8. No differences were seen for any species except for *Strep. agalactiae* from nonclinical-case quarters for which the odds were higher to get a negative mr-PCR result given increasing intertest interval.

DISCUSSION

Culture and mr-PCR Comparative Se and Sp

The mr-PCR technology is more expensive than bacteriological culture. For example, the mr-PCR test used in this study cost around 3 times the price of the culture, besides the mr-PCR instrument and computer. On the other hand, bacteriological culture and identification of bacteria by standard biochemical methods often necessitate more than 48 h to complete, which is not the case with PCR technology, and can be limited by lack of sensitivity, slow-growing or poorly viable organisms, and antimicrobial therapy. A school of thought exists that PCR technology offers potential milk bacteriology diagnostics that are likely to be at least as sensitive as or more sensitive than bacteriological milk culture and more specific. A recent study showed that in approximately half of the clinical mastitis cases in which conventional culture failed to detect bacteria, a mr-PCR test detected mastitis pathogens, often in large quantities (Taponen et al., 2009).

In the present study, the mr-PCR assay tested had estimated Se and Sp values for *Staph. aureus*, *Strep. uberis*, *E. coli*, and *Strep. agalactiae* detection in milk that were often equal to and sometimes higher than with bacteriological culture. On the other hand, the estimated Se of the tested mr-PCR for detecting *Staph. aureus* and *E. coli* in milk from quarters with moderate or severe clinical mastitis were lower than with bacteriological culture. Samples being older when mr-PCR was conducted than when cultured did not account for apparently lower *Staph. aureus* and *E. coli* mr-PCR Se

Table 5. Posterior median and 95% Bayesian credible intervals (BCI) of bacterial species-specific and clinical mastitis severity-specific sensitivity (Se) of bacterial culture and multiplex real-time (mr-PCR) assay using a 1 cfu/0.01 mL culture-positive definition (Dohoo et al., 2011)

Bacteria species	Sample type	mr-PCR ¹		Culture	
		Median Se	95% BCI	Median Se	95% BCI
<i>Staphylococcus aureus</i>	Mild mastitis ²	0.79	(0.70, 0.86)	0.89	(0.86, 0.91)
	Moderate mastitis ³	0.78	(0.67, 0.88)	0.88	(0.85, 0.90)
	Severe mastitis ⁴	0.72	(0.54, 0.86)	0.89	(0.87, 0.92)
<i>Streptococcus uberis</i>	Mild mastitis	0.71	(0.57, 0.83)	0.80	(0.72, 0.86)
	Moderate mastitis	0.84	(0.70, 0.94)	0.78	(0.71, 0.85)
	Severe mastitis	0.89	(0.62, 0.99)	0.83	(0.76, 0.90)
<i>Escherichia coli</i>	Mild mastitis	0.51	(0.37, 0.63)	0.47	(0.35, 0.59)
	Moderate mastitis	0.39	(0.27, 0.52)	0.54	(0.39, 0.67)
	Severe mastitis	0.42	(0.26, 0.59)	0.75	(0.54, 0.89)
<i>Streptococcus agalactiae</i>	Mild mastitis	0.92	(0.65, 0.99)	0.98	(0.94, 0.99)
	Moderate mastitis	0.92	(0.65, 0.99)	0.98	(0.94, 0.99)
	Severe mastitis	0.92	(0.64, 0.99)	0.98	(0.94, 0.99)

¹Polymerase chain reaction threshold cycle (Ct) values for *Staph. aureus*, *Strep. uberis*, *E. coli*, and *Strep. agalactiae* were 28.61, 28.33, 29.55, and 21.52, respectively.

²Abnormal milk only.

³Abnormal milk plus a swollen quarter.

⁴Abnormal milk, swollen quarter, and systemic illness.

(Table 8). A possible explanation is that substances that interfere with 1 or more steps of the mr-PCR assay, but not bacterial culture, are more prominent in milk samples from *Staph. aureus* and *E. coli* IMI with moderate or severe clinical signs. Organic and inorganic chemicals, detergents, antibiotics, enzymes, polysaccharides, fats, proteins, and bacterial debris have been reported as potential PCR inhibitors (Wilson, I. G., 1997). On the other hand, poor viability of or dead bacteria and the presence of antibiotics or disinfectants in milk can also lead to a no-growth culture.

The Se results, but not the Sp results, of mr-PCR and bacteriological culture for detecting bacteria in mammary quarter milk samples were generally different between clinical and nonclinical samples. Because

generally insufficient *Strep. agalactiae* detection events occurred, *Strep. agalactiae* detection is ignored in the following discussion. The mr-PCR assay seems to perform better in nonclinical samples than in clinical samples and, again, a possible explanation could be the presence of mr-PCR-inhibiting substances present in the abnormal milk associated with clinical mastitis.

Multiplex real-time PCR was substantially more sensitive than bacterial culture for detecting *E. coli* in samples from nonclinical mammary quarters. This was true despite the observation that sample age was associated with reduced mr-PCR detection of *E. coli* in this type of sample (Table 7). As results only from samples with ≤ 2 different colony morphologies or bacterial species detected by both methods were modeled, taking

Table 6. Posterior median and 95% Bayesian credible intervals (BCI) of bacterial species-specific and clinical mastitis severity-specific sensitivity (Se) of bacterial culture and multiplex real-time (mr-PCR) assay using a 10 cfu/0.01 mL culture-positive definition (Dohoo et al., 2011)

Bacteria species	Sample type	mr-PCR ¹		Culture	
		Median Se	95% BCI	Median Se	95% BCI
<i>Staphylococcus aureus</i>	Mild mastitis ²	0.92	(0.64, 0.99)	0.72	(0.68, 0.76)
	Moderate mastitis ³	0.91	(0.64, 0.99)	0.72	(0.68, 0.76)
	Severe mastitis ⁴	0.91	(0.63, 0.99)	0.72	(0.68, 0.76)
<i>Streptococcus uberis</i>	Mild mastitis	0.72	(0.57, 0.84)	0.74	(0.66, 0.81)
	Moderate mastitis	0.84	(0.69, 0.94)	0.74	(0.66, 0.81)
	Severe mastitis	0.89	(0.62, 0.99)	0.78	(0.69, 0.85)
<i>Escherichia coli</i>	Mild mastitis	0.52	(0.33, 0.69)	0.33	(0.21, 0.45)
	Moderate mastitis	0.39	(0.23, 0.55)	0.34	(0.21, 0.48)
	Severe mastitis	0.46	(0.25, 0.70)	0.54	(0.31, 0.74)

¹Polymerase chain reaction threshold cycle (Ct) values for *Staph. aureus*, *Strep. uberis*, *E. coli*, and *Strep. agalactiae* were 28.61, 28.33, 29.55, and 21.52, respectively.

²Abnormal milk only.

³Abnormal milk plus a swollen quarter.

⁴Abnormal milk, swollen quarter, and systemic illness.

Table 7. Odds ratios for a positive multiplex real-time PCR test if the duration of sample frozen storage was greater than 100 d versus ≤ 100 d, estimated by sample type in multilevel models with region and season accounted for and herd as random effect

Pathogen	Clinical samples ¹		Nonclinical samples ²	
	Odds ratio	95% CI	Odds ratio	95% CI
<i>Staphylococcus aureus</i>	1.46	(0.77, 2.75)	0.84	(0.47, 1.51)
Season				
Winter	1	—	1	—
Spring	1.13	(0.42, 3.07)	1.84	(0.72, 4.71)
Summer	0.98	(0.30, 3.16)	1.29	(0.36, 4.57)
Fall	1.05	(0.22, 4.97)	2.55	(0.14, 45.26)
Region				
West	1	—	1	—
Ontario	2.71	(1.30, 5.63)	0.77	(0.35, 1.69)
Quebec	3.29	(1.42, 7.63)	—	—
Atlantic	2.01	(0.89, 4.53)	—	—
Random effect: variance (SD)	0.22 (0.47)		0.84 (0.92)	
<i>Streptococcus uberis</i>	0.69	(0.32, 1.47)	0.83	(0.16, 4.30)
Season				
Winter	1	—	1	—
Spring	0.60	(0.20, 1.80)	0.41	(0.04, 4.61)
Summer	0.26	(0.07, 1.01)	0.59	(0.02, 15.17)
Fall	0.13	(0.02, 1.05)	—	—
Region				
West	1	—	1	—
Ontario	0.96	(0.36, 2.56)	0.70	(0.22, 2.24)
Quebec	0.83	(0.27, 2.50)	—	—
Atlantic	1.06	(0.38, 2.94)	—	—
Random effect: variance (SD)	0.72 (0.85)		0.18 (0.42)	
<i>Escherichia coli</i>	0.74	(0.41, 1.33)	0.08	(0.02, 0.47)
Season				
Winter	1	—	1	—
Spring	0.36	(0.16, 0.85)	0.02	(0.00, 0.22)
Summer	0.28	(0.10, 0.77)	0.00	(0.00, 0.10)
Fall	0.11	(0.02, 0.55)	0.01	(0.00, 1.75)
Region				
West	1	—	1	—
Ontario	1.64	(0.82, 3.30)	0.94	(0.30, 3.02)
Quebec	1.53	(0.69, 3.38)	—	—
Atlantic	1.67	(0.80, 3.50)	—	—
Random effect: variance (SD)	0.21 (0.45)		0.91 (0.95)	
<i>Streptococcus agalactiae</i>			0.03	(0.00, 0.23)
Season				
Winter			1	—
Spring			0.01	(0.00, 0.26)
Summer			0.00	(0.00, 0.24)
Fall			—	—
Region				
West				
Ontario				
Quebec				
Atlantic				
Random effect: variance (SD)			1.01 (1.00)	

¹Mammary quarter milk samples from farmer-identified clinical mastitis cases.²Mammary quarter milk samples from randomly selected lactating cows that were apparently not experiencing clinical mastitis.

mr-PCR *E. coli* positive as a diagnosis is less likely to represent a false-positive diagnosis. As expected, the use of a more restricted culture-positive definition lowered the estimated Se of culture, but the gain in Sp was negligible ($\leq 1\%$) making the use of a more restricted culture-positive definition less attractive. For detecting *E. coli* in milk from severe clinical infection cases, the

Se is higher with bacterial culture than with mr-PCR and is also higher than the Se of culture of samples from mild and moderate clinical cases. Whereas the Se estimates vary with degree of clinical severity and with bacterial species, the 95% BCI overlap greatly, indicating that mastitis severity does not affect culture performances.

Table 8. Estimated odds ratio and 95% confidence interval of multiplex real-time PCR (mr-PCR)-positive versus mr-PCR-negative results per frozen storage time lapse means (d) between culture and mr-PCR tests among culture-negative samples

Bacterial species	Clinical ¹		Nonclinical ²	
	Odds ratio	95% CI	Odds ratio	95% CI
<i>Staphylococcus aureus</i>	1.00	(0.99, 1.01)	1.00	(0.99, 1.00)
<i>Streptococcus uberis</i>	1.00	(0.99, 1.00)	1.00	(0.99, 1.01)
<i>Escherichia coli</i>	1.00	(1.00, 1.01)	1.00	(1.00, 1.01)
<i>Streptococcus agalactiae</i>	—	—	0.97	(0.95, 0.99)

¹Mammary quarter milk samples from farmer-identified clinical mastitis cases.

²Mammary quarter milk samples from randomly selected lactating cows that were apparently not experiencing clinical mastitis.

The modification of the final Ct value used to distinguish between positive and negative results and its effect on estimated sensitivities and specificities has not been explored. The comparison of the Ct values against colony-forming-unit numbers appears to be very complex. The quantification of bacterial DNA is a clue to better interpret mr-PCR results and this aspect requires further study.

Errors in the specified prior parameter distributions would introduce error in the Bayesian Se estimates of both tests. Our current best knowledge, of course, is represented by the prior parameters and the observed cross-classification data. The prior Se and Sp estimates we used were derived from Dohoo et al. (2011) in which Se was estimated against the pseudo gold standard of consensus estimates of bacterial species-specific infection status (Andersen et al., 2010). These are suitable prior parameters for our bacterial culture test because they are recent, were derived within the same laboratories using the same protocols by which our culture results were obtained, and were obtained from the same longitudinal cohort of farms (although from a temporally independent sampling series; Reyher et al., 2011). Because no data are available to indicate that bacterial species-specific Se and Sp of bacterial culture of milk samples from clinical mastitis quarters differ from Se and Sp for culture of samples from nonclinical mastitis quarters, the estimates from Dohoo et al. (2011) represent the best available prior knowledge for clinical-case Se and Sp of bacterial culture. To the authors' knowledge, all published reports of the Se and Sp of PCR-based methods in similar contexts are performed directly against bacterial culture. Clearly, these estimates would be biased for use in our context and, thus, unsuitable. We have no objective basis for assuming that definition A prior parameters are more valid than definition C prior parameters. We would simply assume that apparent and estimated Se of bacterial culture for the 4 targeted organisms is greater for definition A than the definition C. In the cases where the estimated

Se of mr-PCR also changed substantially when bacterial culture was interpreted by a different definition and with different priors, it was due to the mr-PCR results being more heavily dependent on priors than on observed cross-classified results of bacterial culture and mr-PCR. Such is the case when the proportion of test-positive results on the tests is not large.

Published data on prior information to be used with mr-PCR are very limited and always relying on a comparison with the bacterial culture as a gold standard. Therefore, a prior distribution reflecting this weak information on mr-PCR test performance was used. A prior distribution slowly increasing to a maximum of 1 represents our current knowledge that the test is reasonably good, with a reluctant prior of seeing the test as perfect. In comparison with uniform priors, the posterior results for the mr-PCR are consistent between the 2 prior distributions, except for *Strep. uberis* subclinical samples and *Strep. agalactiae* (for both definitions A and C). In these cases, uniform priors gave much larger posterior distributions, which reflect the data sparseness of these situations. It should be noted, however, that *E. coli* subclinical samples also had few observations but no differences were seen between the 2 prior distributions used.

How should estimated Se <1.0 for both tests, when both tests have some positive results that are negative on the other test, be interpreted? Three types of errors are possibly operating that could produce discordant test results. The first is variation in the test results that is both random and independent between the tests. Independence of errors is confirmed by all dependence models being nondifferent (<3% difference in estimated Se) from the corresponding independence models. The possible second error operating is unidentified characteristics of the 2 tests that are different between the 2. These would include the biological bases for the tests, the methods and instrumentation used, and the criteria for interpreting the tests. It is these differences that we wish to explore and understand so as to develop

strategies for choosing the best test for a given milk sample-testing context. The final general error possibly contributing to discordant nonpositive test results is differences in the handling of samples before each of the 2 tests. It is for this reason that estimations were made of the effects of sample age and of the difference in sample age between conduct of bacterial culture and mr-PCR.

Freezing Effect

Milk sample preservation before testing may have been a factor that affected our observed results, even if they do not seem to explain differences in estimated Se of the 2 laboratory methods that were studied. During CBMRN NCDF milk sample collection, due to shipping distances to regional bacteriology labs and the need to accommodate the laboratories' weekly sample analysis capacity, freezing of milk samples was necessary. A lot of milk samples taken by farm personnel were frozen and stored on the farm until monthly pickup by technicians. The quality of storage may have been affected, as samples frozen on farms are often placed in the freezer compartment of older refrigerator/freezer units in the milk house or barn. The freezers in these units often do not maintain a constant temperature and may go through frequent and extreme freeze-thaw cycles. Unfortunately, it was not possible to evaluate the effect of this issue on the culture and mr-PCR results. Unpublished data of K. Reyher (University of Prince Edward Island, PEI, Canada) made an analysis of frozen sample storage time that suggested reduced recovery by bacterial culture (all types of samples combined) of *Staph. aureus*, *Strep. uberis*, and *E. coli* when frozen sample storage time was over 100 d. In the present study, the only significant associations of sample age and mr-PCR results were reduced detection of *E. coli* and *Strep. agalactiae* on nonclinical samples. Moreover, the difference in sample age between conduct of bacterial culture and of mr-PCR did not concord with any apparent differences between Se estimates of the 2 tests. On the contrary, estimated Se of mr-PCR for detecting *Strep. agalactiae* might have been higher than the corresponding bacterial culture Se estimates had the samples been closer in age at the time that the tests were conducted (Table 8).

These results should not be applied broadly to all PCR-based diagnostic tests. The various different PCR-based tests available for detecting bacterial species in milk samples may differ from one another in ways that affect test Se or Sp systematically or specifically for certain bacterial species or when applied to different types of samples. However, the results documented in the report may be very suitable as sources of

prior parameter distributions for Bayesian estimation of Se and Sp characteristics of alternative PCR tests. Further work should also delineate in detail the effects of sample storage on performance of PCR-based approaches to detecting bacteria in milk samples.

CONCLUSIONS

The Sp of both bacterial culture and a mr-PCR assay for detecting *Staph. aureus*, *Strep. uberis*, *E. coli*, and *Strep. agalactiae* in milk samples from mammary quarters with clinical signs and mammary quarters with no clinical signs are very high. For detecting *Staph. aureus*, the Se ranged from about 0.66 to 0.96, with bacterial culture being apparently superior in applications to samples from clinically affected quarters. For detecting *Strep. uberis*, the Se ranged from about 0.66 to 0.96, with both tests being equivalent. This mr-PCR is not superior to bacterial culture for detecting *E. coli* from severe clinical cases, but it is in application to non-clinical samples. Further work should be done to extend these results to other PCR-based tests for detecting bacterial species in milk samples and to elucidate the limits of sample handling and storage and the potential existence of substances in clinical case samples that may interfere with PCR reactions.

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